

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



B36

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁷ : C12N 15/12, 15/54, 13/00, 11/02, 9/12, C07K 14/215, 17/02, C12P 19/34</p>	<p>A1</p>	<p>(11) International Publication Number: WO 00/60072 (43) International Publication Date: 12 October 2000 (12.10.00)</p>
<p>(21) International Application Number: PCT/GB00/01289 (22) International Filing Date: 6 April 2000 (06.04.00) (30) Priority Data: 9907813.1 6 April 1999 (06.04.99) GB (71) Applicant (for all designated States except US): MEDICAL BIOSYSTEMS LTD. [GB/GB]; The Old Mill, Beaston Cross, Broadhempston, Nr. Totnes, Devon TQ9 6BX (GB). (72) Inventor; and (75) Inventor/Applicant (for US only): DENSHAM, Daniel, Henry [GB/GB]; The Old Mill, Beaston Cross, Broadhempston, Nr. Totnes, Devon TQ9 6BX (GB). (74) Agent: GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).</p>		<p>(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
<p>(54) Title: POLYNUCLEOTIDE SYNTHESIS USING A PROCESSING ENZYME</p> <p>(57) Abstract</p> <p>A method for polynucleotide synthesis, comprises the steps of: (i) reacting a polynucleotide processive enzyme, e.g. a polymerase or TdT, with a nucleotide substrate under conditions suitable for enzyme activity; and (ii) modulating the conformation of the enzyme, e.g. using radiation, to allow incorporation of a predetermined nucleotide.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

POLYNUCLEOTIDE SYNTHESIS USING A PROCESSING ENZYME

Field of the Invention

This invention relates to a method for polynucleotide synthesis.

Background of the Invention

5 At present the demand for synthetic polynucleotides is large, due in most part to the need for oligonucleotides of known sequence to be used as primers within the Polymerase Chain Reaction (PCR) or within polynucleotide sequencing strategies. More recently, demand has increased even further with the advent of polynucleotide hybridisation arrays. These arrays have, attached to a solid support (or chip), either
10 oligonucleotide probes that hybridise with the sample to be tested, or sample to which labelled oligonucleotide probes can hybridise (Lysov, Dokl. Akad. Nauk SSSR (1988) 303:1508-1511; Bains *et al*, J. Thero. Biol., 135:303-307; Drameanac *et al*, Genomics, 4:114-128). This hybridisation pattern is then used to reconstruct the target polynucleotide sequence. This technique has been further facilitated by the utilisation
15 of light-generated oligonucleotide arrays (Fador *et al*, Proc. Natl. Acad. Sci. USA (1994) 91:5022-5026).

 All current techniques are restricted in the length of synthetic polynucleotide that can be produced and the accompanying problem of low yields. They also employ a significant number of manipulations and hence take a significant period of time to
20 execute.

 There is therefore a need for an improved method for the synthesis of polynucleotides which significantly increases the maximum length of the polynucleotide synthesised and increases the rate at which such a polynucleotide is synthesised. Such a process would preferably be carried out by an automated
25 process, reducing the complexity and cost associated with existing methods.

Summary of the Invention

 The present invention is based on the realisation that electromagnetic radiation can be used to generate conformational changes within a polynucleotide processive enzyme, such that by controlling the radiation applied to such an enzyme, the
30 sequence of the polynucleotide strand produced can be pre-determined. This enables the production of "synthetic" polynucleotides in real-time by manipulating the normal *in vivo* polynucleotide assembly process.

 According to the present invention, a method for synthesising a polynucleotide comprises the steps of:

- (i) reacting a polynucleotide processive enzyme with a nucleotide substrate under appropriate conditions; and
- (ii) exposing the enzyme to a controlled environment (including radiation) so as to affect the three-dimensional conformation of the enzyme and hence determine/affect the sequence of the polynucleotide produced.

Description of the Invention

If radiation is used to control the conformation of the processive enzyme, then it may be applied to a sample using a number of techniques. These include evanescent wave spectroscopy techniques, in particular surface plasmon resonance (SPR) spectroscopy.

The application of radiation to the processive enzyme via the application of laser technology (Light Amplification by Stimulated Emission of Radiation) is particularly applicable to the present invention due to the monochromatic and controllable nature of the radiation produced by such devices.

The control of the conformational structure of processive enzymes can be accomplished by controlling the environment in which they act. It has been shown that variations in such conditions as pH and salt content/concentration of the reaction medium can have an effect on the three-dimensional structure and hence on the activity of such enzyme systems (Wong *et al*, Biochemistry (1991) 30:526-537).

The addition of the specified nucleotide, and hence the synthesis reaction, may be accomplished by directly creating the ability of the processive enzyme to undergo a conformational change that is specific for the addition of a particular nucleotide, depending on the form of radiation delivered. This could be achieved by engineering (via state-of-the art genetic manipulation techniques) a processive molecule (or molecule associated with it) such that it contained a chemical/moiety/peptide group or groups that enable the molecule to convert or transduce radiation into a conformational change. These chemical/moiety group or groups may be so positioned so as to select for the nucleotide to be added to the growing polynucleotide chain. The method may therefore proceed on a "real-time" basis, to achieve a high rate of polynucleotide synthesis.

The present method for the synthesis of a polynucleotide, as indicated above, involves the control of the environment in which a polynucleotide processive enzyme is placed, and hence of the three-dimensional conformation of said enzyme. This

three-dimensional conformation in turn selects if and/or which substrate nucleotide is added to the growing polynucleotide strand.

The term "polynucleotide" is used herein as to be interpreted broadly, and includes DNA and RNA, including modified DNA and RNA, as well as other hybridising nucleic acid-like molecules, e.g. peptide nucleic acid (PNA).

The term "polynucleotide processive/polymerisation enzyme" is used herein as to be interpreted broadly, and pertains to ubiquitous proteins that can attach one nucleotide to another in order to create a polynucleotide. Such a group will, of course, include all polymerases, both DNA- and RNA-dependant and also such enzyme groups as terminal deoxynucleotidyl transferases (Kato *et al*, J. Biol. Chem., (1967) 242:2780; & Frohman *et al*, Proc. Natl. Acad. Sci. USA, (1988) 85:8998).

Using a polynucleotide processive enzyme in order to control the synthesis of a polynucleotide offers several advantages for the success of this method. Firstly, the problem of reaction yield in solid phase synthesis is avoided due to the highly efficient catalytic nature of organic molecules. Secondly, speed of synthesis and polynucleotide strand length are several orders of magnitude greater than those currently available, again due to the requirements of the enzyme systems in their native environments.

Another important aspect of the invention is the realisation that, although a large number of polynucleotide processive enzymes require an existing polynucleotide template to initiate polynucleotide synthesis in their native environment/form, this is not always the case. As the effectiveness of the nucleotide (Crick-Watson) base pairing and hence of complementary strand construction is ultimately dependent on the three-dimensional conformation (and resulting kinetic parameters) of the processive enzyme, this system can be disrupted and utilised in order to externally control the sequence of nucleotides polymerised. In the specific case of the utilisation of polymerases for the present invention, therefore, the "synthetic" polynucleotide strand produced may not (and in most instances will not) be a complementary copy of the template polynucleotide strand. Disruptions to polymerase function via active site mutation are known in the art (Freemont *et al*, Proteins (1986) 1:66-73) but, critically, they are not conformationally/spatially modulated. Such disruption/mutation could take the form, as in the present invention, of a reduction in the natural fidelity of the polymerase such that it does not discriminate against dideoxynucleotides. This would allow the mutated polymerase to insert any nucleotide in solution into the growing

polynucleotid chain independently of the nucleotid sequence of the polynucleotide template. The nature of such binding site modifications that are fixed upon molecular cloning (i.e. not capable of external real-time conformational modulation) are known in the art (Ollis *et al*, Nature (1985) 313:762-766 & Freemont *et al*, Proteins (1986) 1:66-73) and are directed at the polymerase active site. For example, it has been shown that Phe⁷⁶² of *E. Coli* polymerase I is one of the amino acids that directly interact with the substrate nucleotides (Joyce *et al*, Ann. Rev. Biochem. (1994) 63:777-822 & Astake *et al*, J. Biol. Chem. (1995) 270:1945-54). Converting this amino acid to a Try results in a mutant DNA polymerase that does not discriminate against dideoxynucleotides. See US-A-5614365 and copending U.S. Application No. 08/525,087, of Deb K. Chatterjee, filed September 8, 1995, entitled "Mutant DNA Polymerases and the Use Thereof", which are expressly incorporated herein by reference.

These modifications have since been characterised further in order to define polymerases with reduced error rate, that is reduced misincorporation of nucleotides during nucleic acid synthesis and/or increased fidelity of polymerisation. See WO-A-99/10366, which is expressly incorporated herein by reference. This application relates to a method of making such high fidelity polymerases by modifying or mutating the nucleotide binding domain of the polymerase (e.g. the O-helix).

An important aspect of the method of the present invention is the use of a protein/peptide/chemical group/moiety that has a structure/conformation capable of being modulated via interaction with photons and/or energy derived from photons. Such groups include, but are not limited to, biological molecules which transduce photonic energy, synthetic dye compounds, and energy-absorbing chemical groups. A preferred embodiment of the present invention involves the utilisation of biological photonic transducers to modulate the polymerase active site (e.g. O-helix) conformation and hence polymerase activity. This group of biological transducers includes, but is not limited to, light-harvesting (LH) complexes/molecules and systems involved photosynthesis (e.g. bacterial complexes such as LH1 and LH2; see Papiz *et al*, Trends Plant Sci. (1996) 1:198-206), direct photon-driven proton pump complexes/subunits (e.g. bacteriorhodopsin (BR) from the purple membrane of *Halobacterium salinarum*; see Oka *et al*, Biophys. J. (1999) 76:1018-1023), sensory pigments, (e.g. retinal and associated protein complexes) and natural fluorescent

proteins and their engineered derivatives (e.g. Green Fluorescent Protein (GFP); Heim *et al*, Proc. Natl. Acad. Sci. USA (1994) 91:12501-12504).

The active sites of polymerase molecules which affect overall function and are targeted for controlled conformational modulation within the present invention, include, but are not restricted to, the O-Helix, the K-helix, and the inter O-P loop of Taq DNA polymerase or analogous positions in other polymerases; see WO-A-98/40496.

Methods for genetically "fusing" the sequences and hence structures of two or more peptides/proteins are well known in the art and have been applied extensively in the case of Green Fluorescent Protein (GFP) to construct fused mutant or "chameleon" proteins to create fluorescent labels for specific substrates such as Ca^{2+} and to modulate spectral response (Heim *et al*, Proc. Natl. Acad. Sci. USA (1994) 91:12501-12504 & Heim *et al*, Nature (1997) 388:882-887).

In a preferred embodiment, the O-helix of T7 polymerase is fused to a fluorescent mutant of GFP. This results in a fusion protein whose nucleotide substrate affinities can be modulated in response to exposure to differing wavelengths of light and the sub-type of GFP mutation chosen.

In a further preferred embodiment, the photon-transducing protein and the polymerase are cloned separately and reactive side groups capable of taking part in cross-linking reaction(s) are site-selectively introduced into each protein structure at the desired location (e.g. the O-helix within the polymerase).

A number of strategies may be used to attach reactive groups to the proteins. Strategies include, but are not limited to, the use of site-directed mutagenesis and unnatural amino acid mutagenesis (Anthony-Cahill *et al*, (1989) Trends Biochem. Sci. 14:400) to introduce cysteine and ketone handles to act as a site for cross-linking to occur. Cross-linking reagents which contain two reactive groups can then be employed to covalently link the chosen side groups (Haugland, Handbook of Fluorescent Probes and Research Chemicals, 6th Edition, Molecular Probes, p94-106). Examples of such cross-linking reactions include thio(derived from cysteine)-thiol cross-linking, amine-amine cross-linking, amine-thiol cross-linking, amine-carboxylic acid cross-linking, amine-carbohydrate cross-linking and thiol-carbohydrate cross-linking.

As already outlined, it is foreseen that in some circumstances the presence of an existing polynucleotide strand may not be necessary for template-directed synthesis to take place at all. For example, this would be possible using extensively

modified polymerases that have been cloned to "design" via state-of-the-art recombinant genetic techniques. As stated previously, the conformation of these polymerases would be under external control (preferably a radiation source) and this external manipulation of the enzyme's nucleotide substrate specificity determines the growing polynucleotide's polymerisation sequence. Moreover, certain groups of polynucleotide synthetic enzymes do not require starting polynucleotide templates for synthesis, even in their "native" environment. Such a group is the terminal deoxynucleotidyl transferase group of enzymes. Terminal deoxynucleotidyl transferase (TdT) catalyses the repetitive addition of mononucleotides from a deoxynucleoside triphosphate to the terminal 3'-hydroxy of a DNA initiator, with the release of inorganic phosphate. The enzyme requires an oligodeoxynucleotide containing at least three phosphate groups and a free 3'-OH to serve as initiator.

In a further embodiment of the invention, therefore, a free 3'OH group extending from a solid support will act as an initiator for the TdT and the engineered enzyme will synthesise a polynucleotide via the addition of substrate nucleotides via the control of radiation applied to the enzyme. In a simpler, but slower, embodiment of this system, the enzyme could be made (via genetic engineering or control of reaction conditions) to polymerise any nucleotide available as substrate and hence control of the nucleotide present in solution would determine the sequence of the polynucleotide synthesised.

In another embodiment of the invention, the TdT or polymerase (or any other polynucleotide polymerase) is bound to a solid support and the nucleotides and/or radiation are made available to the enzyme whilst bound. This embodiment allows the user to localise the application of radiation and/or substrate and grow the new polynucleotide strand into solution. This configuration of the invention has the added advantage that, once the desired polynucleotide has been synthesised, it can be released from the bound enzyme and the process begun again (i.e. it is a regenerative process).

A preferred embodiment of the invention involves the localisation of the polynucleotide processive/polymerase enzyme system in space. This localisation may take the form of, but is not restricted to, immobilisation on a solid support. Localisation of the polymerase in space offers several important advantages for the success of this method. Firstly, the problem of unwanted attenuation of the applied radiation/control environment is reduced as the exact location of the polymerase

in space is known and can hence be more easily selectively controlled via localised environmental modulation (e.g. laser pulses). Secondly, unwanted/uncontrolled interaction (or random energy attenuation) of the enzyme system with the local environment/substrate (e.g. nucleotides) not directly involved with the polymerase is reduced considerably. This is particularly relevant if radiation (e.g. photonic) is utilised, as envisioned within the scope of the invention, to control/attenuate the conformational form of the polymerase.

Immobilisation may be carried out using standard procedures known in the art. In particular, immobilisation using standard amine coupling procedures may be used, with attachment of ligand-associated amines to, say, a dextran or N-hydroxysuccinimide ester-activated surface. In a preferred embodiment of the invention, the polymerase is immobilised onto a SPR sensor chip surface where changes in the refractive index may be measured. Examples of procedures used to immobilise biomolecules to optical sensors are disclosed in EP-A-0589867, and Löffas *et al*, *Biosens. Bioelectron.* (1995) 10: 813-822.

Localisation within space can also be carried out, and is a further embodiment of the invention, via the utilisation of a Laser Tweezer or Optical Trap System (Sheetz, Ed., *Laser Tweezers in Cell Biology*, Vol.55 of *Methods in Cell Biology* (Academic Press, New York, 1997)). Optical Tweezers exploit the fact that light exerts force on matter. Dielectric particles, such as uniform beads or bacterial cells, are attracted to and trapped near the waist of a laser beam that has been focused through a microscope objective. Applied forces will displace a trapped bead from the trap centre, with a linear dependence of displacement on force. Biological molecules such as polymerases, as within an embodiment of the present invention, can be bound to polystyrene or silica beads, which are usually $\sim 1 \mu\text{m}$ in diameter. The trap can then be used to steer the immobilised polymerase into the desired experimental geometry/controlled environment within the reaction flow cell.

The polynucleotide polymerisation enzyme used in the invention may be of any known type. For example, a polymerase may be any DNA-dependant DNA polymerase, e.g. T7 gene 5 polymerase or Taq polymerase. If the target polynucleotide is an RNA molecule, then the polymerase may be an RNA-dependent DNA polymerase, i.e reverse transcriptase or a RNA-dependent RNA polymerase, i.e. RNA replicase. TdT is preferred.

Nuclear Magnetic Resonance (NMR) Spectroscopy (Bradley *et al*, J. Mol. Biol., (1990) 215:607-622) and Electron Paramagnetic Resonance (EPR) Spectroscopy (Todd *et al*, Biochemistry, (1991) 30:5515-5523) are further preferred methods of subjecting the polynucleotide polymerisation enzyme to specific types of radiation to control specific nucleotide addition via conformational control and at the same time allow structural/conformational data feedback. Using this technique it is also possible to measure the response of the enzyme molecules. NMR spectroscopy measures the magnetic properties of compounds. Nuclei of compounds are energetically orientated by a combination of applied magnetic field and radio-frequency radiation. When the energy exerted on a nucleus equals the energy difference between spin states (the difference between orientation parallel or anti-parallel to the direction of the applied fields), a condition known as resonance is achieved. The absorption and subsequent emission of energy associated with the change from one spin state to the other, are detected by a radio-frequency receiver.

In yet another embodiment of the invention, the starting 3'OH group is attached to a bead (e.g. one end of the biotin could be biotinylated and attached to a streptavidin-coated polystyrene sphere; Chu *et al*, Optical Society of America, Washington, DC, (1990), 8:202) and held within an optical trap (Ashkin *et al*, Opt. Lett. (1986) 11:288) within a flow cell (as outlined previously). As the polynucleotide processive enzyme (under external control) synthesises new polynucleotide, this new polynucleotide can be moved in space via the optical trap (or also known as optical tweezers) and hence keep the processive enzyme within the field of detection. It is also envisaged that this system could work in the reverse set-up with the bound polynucleotide processive enzyme being held by the optical trap.

The following Example illustrates the invention.

Example

The following analysis was carried out on a modified BIAcore® 2000 system (Biacore AB, Uppsala, Sweden) with a sensor chip CM5 (Research grade, Biacore AB) as the optical sensor/control/reaction surface. The instrument was provided with an integrated μ -fluidic cartridge (IFC) which allows analysis in four cells by a single sample-injection.

Preparation of Cysteine-Tagged Bacteriorhodopsin

Bacteriorhodopsin (BR) is a light-driven proton pump in the purple membrane of *Halobacterium salinarum*. The photocycle of BR is initiated by absorption of a

photon by the retinal chromophore. The site-specific mutation Ile²²² → Cys (cysteine mutation) was introduced (Erlanson *et al*, Tetrahedron (1997) 53:12041) into the bop gene. According to the current structural model of bacteriorhodopsin (Lanyi *et al*, Science (1999) 286:255-260), Ile²²² is located at the cytoplasmic end of helix G.

5 X-Ray Diffraction studies (Lanyi *et al*, Science (1999) 286:255-260) and Heavy Atom Labelling (Lanyi *et al*, Biophys. J. (1999) 76:1018-1023) show major structural/conformational changes within helix G associated with photonic absorption. The changed bop gene was constructed by inserting it into a non-integrating vector, with novobiocin resistance as the selective marker. *Halobacterium salinarum* was

10 transformed as described by Ni *et al*, Gene (1990) 90:169-172 & Needleman *et al*, J. Biol. Chem. (1991) 266:11478-11484. The mutated protein was purified from *H. salinarum* as purple membrane (PM) sheets according to the standard method described by Oesterhelt and Stoekenius., Methods Enzymol. (1974) 31:667-678.

Preparation of Cysteine-Tagged T7 Polymerase

15 An expression vector containing T7 polymerase coding sequence was constructed. A site-specific mutation was introduced (Erlanson *et al*, Tetrahedron (1997) 53:12041) into the O-helix coding region at Arg⁵¹⁸ → Cys⁵¹⁸. Cell pellets were lysed with a French press, and the enzyme was purified by Ni-nitrilotriacetic acid affinity chromatography, followed by cation exchange chromatography (sulfopropyl-

20 Sepharose fast flow) and a final step of size-exclusion chromatography (Superdex 200).

Thiol-Thiol Cross-linking Reaction

10 µM of mutated T7 and 10 µM of mutated bacteriorhodopsin were added to Hepes (10 mM Hepes, 150 mM NaCl, 0.05% surfactant P20 (BIAcore AB, Uppsala,

25 Sweden), pH 7.4) buffer solution containing 2 mM β-mercaptoethanol (which was added to increase the specificity of cross-linking). After a 2-hour incubation at 25°C, the cross-linking reaction was quenched by the addition of a thiol-capping reagent, methyl methanethiolsulfonate (20 mM), and the products were confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The

30 bacteriorhodopsin-polymerase complex was then purified by anion-exchange chromatography on Mono-Q and then resuspended in Hepes Buffer (complex at 8 mg/ml).

Immobilisation of the Bacteriorhodopsin-Polymerase Complex

Immobilisation of the bacteriorhodopsin-polymerase to the sensor chip was carried out according to Jönsson *et al*, *Biotechniques* (1991); 11:620-627. Briefly, the sensor chip environment was equilibrated with Hepes buffer (10 mM Hepes, 150 mM NaCl, 0.05% surfactant P20 (BIAcore AB, Uppsala, Sweden), pH 7.4). Equal volumes of N-hydroxysuccinimide (0.1 M in water) and N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) (0.1 M in water) were mixed together and injected across the chip (CM5) surface, to activate the carboxymethylated dextran. The bacteriorhodopsin-polymerase was mixed with 10 mM sodium acetate (100 μ l, pH 5) and injected across the activated surface. Finally, residual N-hydroxysuccinimide esters on the sensor chip surface were reacted with ethanolamine (35 μ l, 1 M in water, pH 8.5), and non-bound bacteriorhodopsin-polymerase was washed from the surface. The immobilisation procedure was performed with a continuous flow of Hepes buffer (5 μ l/min) at a temperature of 25°C.

Oligonucleotides

The non-active target and primer oligonucleotides defined as SEQ ID No. 1 and SEQ ID No. 2 in WO-A-99/05315 were used. The two polynucleotides were reacted under hybridising conditions to form the target-primer complex.

The primed DNA was then suspended in buffer (20 mM Tris-Hcl, pH 7.5, 8 mM MgCl₂, 4% (v/v) glycerol, 5 mM dithiothreitol (DDT), 40 mg bovine serum albumin) containing 60 mM carbonyldiphosphate (to maintain complex integrity) and 80 mM thioredoxin and injected over the chip surface and allowed to bind to the bacteriorhodopsin-polymerase complex via the formation of a bacteriorhodopsin/polymerase/thioredoxin/DNA complex.

DNA Synthesis

This step was carried out using the apparatus shown in Fig. 1 of WO-A-99/05315, but using only one focusing assembly (5) for pulsing monochromatic light into the cell.

The first desired nucleotide to be part of the newly synthesised polynucleotide is introduced into the fluidic cell (6) at a flow rate of 30 μ l/min, at a temperature of 25°C and a data collection rate of 10Hz. As the nucleotides pass the focusing assembly (5), monochromatic light is tuned across the wavelength band 300-600 nm (via a solid-state diode tunable laser) whilst the SPR signal is monitored. Once the SPR signal indicates that conformational nucleotide addition has taken place, the

wavelength of the applied laser pulse is maintained. Then HEPES buffer only is allowed to flow over the chip surface for 10 seconds at a flow rate of 30 $\mu\text{L}/\text{min}$ to remove unreacted nucleotides. Subsequently, the next desired nucleotide is added and the cycle repeated for the desired length of polynucleotide.

- 5 Alternatively, all nucleotides may be injected into the flow cell at once at a flow rate of 30 $\mu\text{L}/\text{min}$ and the incident monochromatic laser light attenuated across the wavelength range 300-600 nm such that the desired nucleotides are added in the desired sequence.

CLAIMS

1. A method for polynucleotide synthesis, comprising the steps of:
 - (i) reacting a polynucleotide processive enzyme with a nucleotide substrate under conditions suitable for enzyme activity; and
 - 5 (ii) modulating the conformation of the enzyme to allow incorporation of a predetermined nucleotide.
2. A method according to claim 1, wherein the conformation is modulated by surface plasmon resonance.
3. A method according to claim 1, wherein the conformation is modulated by a
10 laser.
4. A method according to any preceding claim, wherein the conformation is modulated by applied radiation.
5. A method according to claim 4, wherein the enzyme is fixed within the field of applied radiation.
- 15 6. A method according to any preceding claim, wherein the enzyme is immobilised on a solid support.
7. A method according to any preceding claim, wherein the enzyme is a polymerase.
8. A method according to any of claims 1 to 6, wherein the enzyme is a terminal
20 deoxynucleotidyl transferase.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 00/01289

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/54 C12N13/00 C12N11/02 C12N9/12
C07K14/215 C07K17/02 C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, PAJ, CAB Data, STRAND, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 05315 A (DENSHAM DANIEL HENRY ;MEDICAL BIOSYSTEMS LTD (GB)) 4 February 1999 (1999-02-04) cited in the application the whole document	1-8
A	WO 98 40496 A (PERKIN ELMER CORP) 17 September 1998 (1998-09-17) cited in the application page 7, line 21 -page 8, line 7	
A	WO 99 10366 A (LIFE TECHNOLOGIES INC) 4 March 1999 (1999-03-04) cited in the application the whole document	
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

9 August 2000

Date of mailing of the international search report

17/08/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 851 epo nl,
Fax: (+31-70) 340-3018

Authorized officer

Hornig, H

INTERNATIONAL SEARCH REPORT

Inter national Application No

PCT/GB 00/01289

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 09451 A (CHATTERJEE DEB K ; HUGHES A JOHN JR (US)) 13 March 1997 (1997-03-13) the whole document	
A	EP 0 727 496 A (HARVARD COLLEGE) 21 August 1996 (1996-08-21) the whole document	
A	EP 0 655 506 A (HARVARD COLLEGE) 31 May 1995 (1995-05-31) the whole document	
A	WO 97 26368 A (MUELLER MANFRED W) 24 July 1997 (1997-07-24) the whole document	
A	EP 0 669 395 A (BOEHRINGER MANNHEIM GMBH) 30 August 1995 (1995-08-30) the whole document	
A	WO 90 05303 A (PHARMACIA AB) 17 May 1990 (1990-05-17) the whole document	

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 00/01289

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9905315 A	04-02-1999	AU 8455998 A EP 1017848 A	16-02-1999 12-07-2000
WO 9840496 A	17-09-1998	AU 6466998 A EP 0983364 A	29-09-1998 08-03-2000
WO 9910366 A	04-03-1999	AU 9123598 A EP 1012161 A	16-03-1999 28-06-2000
WO 9709451 A	13-03-1997	US 5939301 A AU 7236296 A EP 0871775 A JP 2000502882 T US 5948614 A US 6015668 A	17-08-1999 27-03-1997 21-10-1998 14-03-2000 07-09-1999 18-01-2000
EP 0727496 A	21-08-1996	US 5614365 A DE 29513622 U DE 29513639 U JP 2691149 B JP 8205874 A AT 143057 T AU 4193396 A CA 2201885 A CN 1170439 A DE 69400567 D DE 69400567 T DE 655506 T DK 655506 T EP 0655506 A ES 2072238 T FI 971611 A GR 95300040 T HU 77037 A WO 9612042 A ZA 9508761 A	25-03-1997 19-10-1995 19-10-1995 17-12-1997 13-08-1996 15-10-1996 06-05-1996 25-04-1996 14-01-1998 24-10-1996 06-02-1997 28-09-1995 10-03-1997 31-05-1995 16-07-1995 13-06-1997 31-07-1995 02-03-1998 25-04-1996 13-05-1996
EP 0655506 A	31-05-1995	US 5614365 A AT 143057 T AU 4193396 A CA 2201885 A CN 1170439 A DE 29513622 U DE 29513639 U DE 69400567 D DE 69400567 T DE 655506 T DK 655506 T EP 0727496 A ES 2072238 T FI 971611 A GR 95300040 T HU 77037 A JP 2691149 B JP 8205874 A WO 9612042 A ZA 9508761 A	25-03-1997 15-10-1996 06-05-1996 25-04-1996 14-01-1998 19-10-1995 19-10-1995 24-10-1996 06-02-1997 28-09-1995 10-03-1997 21-08-1996 16-07-1995 13-06-1997 31-07-1995 02-03-1998 17-12-1997 13-08-1996 25-04-1996 13-05-1996

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter national Application No

PCT/GB 00/01289

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9726368 A	24-07-1997	DE 19601385 A EP 0874914 A JP 11503330 T	17-07-1997 04-11-1998 26-03-1999
EP 0669395 A	30-08-1995	DE 4406524 A JP 2777550 B JP 7270425 A US 5573913 A	31-08-1995 16-07-1998 20-10-1995 12-11-1996
WO 9005303 A	17-05-1990	SE 462454 B AT 136651 T DE 68926255 D DE 68926255 T EP 0589867 A JP 2815120 B JP 4501605 T SE 8804073 A US 5436161 A US 5242828 A	25-06-1990 15-04-1996 15-05-1996 31-10-1996 06-04-1994 27-10-1998 19-03-1992 10-11-1988 25-07-1995 07-09-1993